



THE UNIVERSITY *of* EDINBURGH

## Edinburgh Research Explorer

# Neutrophil Extracellular Traps Exhibit Antibacterial Activity against *Burkholderia pseudomallei* and Are Influenced by Bacterial and Host Factors

### Citation for published version:

Riyapa, D, Buddhisa, S, Korbsrisate, S, Cuccui, J, Wren, BW, Stevens, MP, Ato, M & Lertmemongkolchai, G 2012, 'Neutrophil Extracellular Traps Exhibit Antibacterial Activity against *Burkholderia pseudomallei* and Are Influenced by Bacterial and Host Factors', *Infection and Immunity*, vol. 80, no. 11, pp. 3921-3929.  
<https://doi.org/10.1128/IAI.00806-12>

### Digital Object Identifier (DOI):

[10.1128/IAI.00806-12](https://doi.org/10.1128/IAI.00806-12)

### Link:

[Link to publication record in Edinburgh Research Explorer](#)

### Document Version:

Publisher's PDF, also known as Version of record

### Published In:

*Infection and Immunity*

### General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

### Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



# Neutrophil Extracellular Traps Exhibit Antibacterial Activity against *Burkholderia pseudomallei* and Are Influenced by Bacterial and Host Factors

Donporn Riyapa,<sup>a</sup> Surachat Buddhisa,<sup>a</sup> Sunee Korbsrisate,<sup>b</sup> Jon Cuccui,<sup>c</sup> Brendan W. Wren,<sup>c</sup> Mark P. Stevens,<sup>d</sup> Manabu Ato,<sup>e</sup> and Ganjana Lertmemongkolkhai<sup>a</sup>

The Centre for Research & Development of Medical Diagnostic Laboratories, Faculty of Associated Medical Sciences, Khon Kaen University, Khon Kaen, Thailand<sup>a</sup>; Department of Immunology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand<sup>b</sup>; London School of Hygiene and Tropical Medicine, London, United Kingdom<sup>c</sup>; Roslin Institute and Royal (Dick) School of Veterinary Studies, University of Edinburgh, Edinburgh, United Kingdom<sup>d</sup>; and Department of Immunology, National Institute of Infectious Diseases, Tokyo, Japan<sup>e</sup>

*Burkholderia pseudomallei* is the causative pathogen of melioidosis, of which a major predisposing factor is diabetes mellitus. Polymorphonuclear neutrophils (PMNs) kill microbes extracellularly by the release of neutrophil extracellular traps (NETs). PMNs play a key role in the control of melioidosis, but the involvement of NETs in killing of *B. pseudomallei* remains obscure. Here, we showed that bactericidal NETs were released from human PMNs in response to *B. pseudomallei* in a dose- and time-dependent manner. *B. pseudomallei*-induced NET formation required NADPH oxidase activation but not phosphatidylinositol-3 kinase, mitogen-activated protein kinases, or Src family kinase signaling pathways. *B. pseudomallei* mutants defective in the virulence-associated Bsa type III protein secretion system (T3SS) or capsular polysaccharide I (CPS-I) induced elevated levels of NETs. NET induction by such mutants was associated with increased bacterial killing, phagocytosis, and oxidative burst by PMNs. Taken together the data imply that T3SS and the capsule may play a role in evading the induction of NETs. Importantly, PMNs from diabetic subjects released NETs at a lower level than PMNs from healthy subjects. Modulation of NET formation may therefore be associated with the pathogenesis and control of melioidosis.

Melioidosis is caused by the motile Gram-negative facultative intracellular pathogen *Burkholderia pseudomallei* and is endemic in Southeast Asia and northern Australia. Melioidosis can establish with a variety of clinical features, ranging from acute fulminant septicemia to chronic localized infection. The case fatality rate of patients with severe melioidosis is approximately 50% in Thailand (7, 16, 31, 39). *B. pseudomallei* infection often affects individuals with one or more underlying predisposing conditions associated with impaired immune responses, with the major risk factor being diabetes mellitus (DM) (18, 25). There has been much scientific interest in understanding *B. pseudomallei*-host interactions at the cellular level, as the organism is resistant to many antibiotics and no licensed vaccine exists.

Polymorphonuclear neutrophils (PMNs) are highly specialized effector cells involved in host inflammatory responses and immune surveillance (22) and play a key role in control of melioidosis in a murine model (10). A novel antimicrobial activity of PMNs in which the leukocytes generate neutrophil extracellular traps (NETs) was recently reported. Upon activation, PMNs can release NETs composed of chromatin decorated with granular proteins. NETs possess an antimicrobial activity that can entrap and damage microbes extracellularly, and it is believed that they may result from activation of a novel cell death pathway termed NETosis (4, 5, 13, 34, 35).

The pathways leading to release of NETs are ill defined, but it has been reported to require activation of one or more of the phagosomal membrane-bound NADPH oxidase enzyme complexes found in professional phagocytes and B lymphocytes (13). These enzymes catalyze the production of superoxide anions ( $O_2^-$ ), which in turn produce reactive oxygen species (ROS), including dioxygen and hydrogen peroxide ( $H_2O_2$ ), which cause

oxidative damage to microorganisms in the phagosome (2, 13). PMNs from neonates fail to form NETs when activated by inflammatory stimuli and consequently exhibit impaired bactericidal activity against extracellular bacteria (13, 41). A variety of different proinflammatory agonists have been shown to activate NET formation, including the mitogen phorbol myristate acetate (PMA), bacterial lipopolysaccharide (LPS), and the CXC family chemokine interleukin 8 (IL-8) (4, 13). Such agonists have been reported to act in activated PMNs via a chemokine receptor pathway (20); however, the downstream signaling leading to NETosis is not well understood. Src family kinases and p38 mitogen-activated protein kinases (MAPKs) are activated following stimulation with IL-8. There is also evidence that IL-8 stimulates phosphatidylinositol-3 kinase (PI3K) activity in human PMNs (23, 24, 32).

The interaction of *B. pseudomallei* with host cells is known to be influenced by a bacterial type III secretion system (T3SS), encoded by the *bsa* locus. *B. pseudomallei* mutants lacking components of the Bsa secretion and translocation apparatus, including *bsaZ*, *bsaQ*, *bipB*, and *bipD*, are impaired in invasion of epithelial cells, intracellular net replication, survival in macrophages, escape from endocytic vacuoles, and virulence in mice (28–30). Addi-

Received 9 August 2012 Accepted 18 August 2012

Published ahead of print 27 August 2012

Editor: S. R. Blanke

Address correspondence to Ganjana Lertmemongkolkhai, g.lert@yahoo.co.uk.

Supplemental material for this article may be found at <http://iai.asm.org/>.

Copyright © 2012, American Society for Microbiology. All Rights Reserved.

doi:10.1128/IAI.00806-12

TABLE 1 Bacterial strains used in this study

Strain	Description <sup>a</sup>	Source or reference
<i>B. pseudomallei</i> 10276	Wild-type strain isolated from a human melioidosis patient in Bangladesh	Ty Pitt, HPA; Stevens et al. (29)
<i>B. pseudomallei</i> K96243	Wild-type strain isolated from a human melioidosis patient in Thailand	S. Songsivilai, Mahidol, University; Holden et al. (15)
<i>B. pseudomallei</i> 10276 <i>bsaZ</i> ::pDM4	Bsa T3SS structural component mutant; reduced intracellular replication, absence of membrane protrusion formation, and delayed ability to escape endocytic vesicles	Stevens et al. (29)
<i>B. pseudomallei</i> K96243 <i>bsaQ</i> ::pKNOCK-Cm	Bsa T3SS structural component mutant; reduced plaque and MNGC formation, reduced invasion of nonphagocytic cells	Muangsoombut al. (21)
<i>B. pseudomallei</i> K96243 <i>wcbN</i> ::mini-Tn5km2	Transposon mutant, attenuated in BALB/c mouse infection model but epitope to CPS-I-specific antibody is maintained	Cuccui et al. (8)
<i>B. pseudomallei</i> K96243 <i>wcbB</i> ::mini-Tn5km2	Transposon mutant lacking CPS-I, attenuated in BALB/c mouse infection model	Cuccui et al. (8)

<sup>a</sup> MNGC multinucleated giant cells; CPS-I, capsular polysaccharide I.

tionally, the Bsa T3SS influences the virulence of *Burkholderia mallei* (33). A polysaccharide capsule encoded by the *wcb* operon also plays a pivotal role in the pathogenesis of murine melioidosis (37). It has previously been reported that a polysaccharide capsule protects *Streptococcus pneumoniae* against entrapment in NETs (38); however, the role of capsule and of the Bsa T3SS in interactions with human PMN has received little study.

Here, we investigated that role of NETs in the innate response of human PMNs to *B. pseudomallei* and of bacterial virulence factors in counteracting such responses. As we have previously discovered that PMNs from diabetic subjects have impaired antibacterial functions (6), we also explored the possibility that NET formation is altered or impaired in PMNs from DM subjects.

(This work was presented in part at the VI World Melioidosis Congress, 30 November to 2 December 2010, Townsville, Queensland, Australia.)

## MATERIALS AND METHODS

**PMN isolation.** Human PMNs were isolated from fresh heparinized venous blood from healthy and diabetic subjects using the previously reported criteria and methods (6). Permission was obtained from the Khon Kaen University Ethics Committee for Human Research, number HE470506. Briefly, cells were isolated by 3.0% dextran T-500 sedimentation and separated by Ficoll-Hypaque density gradient centrifugation (Sigma), followed by hypotonic lysis to remove residual erythrocytes. Purity was >95%, as measured by differential count following Giemsa staining, and viability was >99%, as determined by trypan blue exclusion.

**Bacterial strains.** *B. pseudomallei* wild-type (WT) strain K96243 is the prototype strain whose genome has been sequenced (15), and WT strain 10276 was isolated from a fatal case of human melioidosis in Bangladesh (29). The 10276 *bsaZ* and K96243 *bsaQ* mutant strains lacking the function of the Bsa T3SS have been described elsewhere (21, 29). We also used K96243 *wcbB* and *wcbN* mutants lacking enzymes required for capsule synthesis as described previously (8). *B. pseudomallei* WT strains K96243 and 10276 were grown in Luria-Bertani (LB) broth, whereas type III secretion and capsule mutants were grown in LB broth containing chloramphenicol and kanamycin, respectively. The number of viable bacteria used was determined by retrospective plating of serial 10-fold dilutions on LB agar plates. The details of the bacteria used in this study are summarized in Table 1.

**Quantification of NET release.** PMNs were incubated for 90 min with *B. pseudomallei* WT, mutant strains, or killed *B. pseudomallei* at a multi-

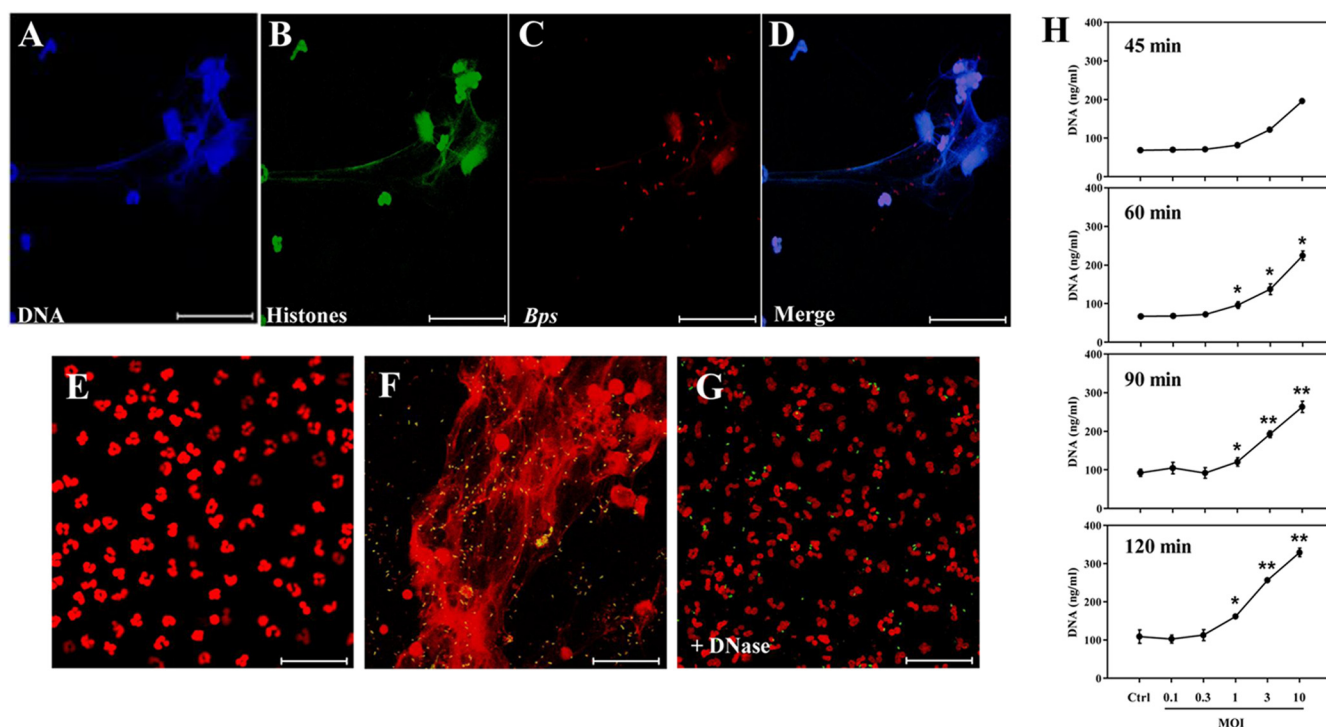
plicity of infection (MOI) of 10. Typically, the number of bacteria used for inoculation of 7 log<sub>10</sub> PMN cells was 8 log<sub>10</sub> CFU. As a positive control, PMNs were separately treated with 100 nM PMA (Sigma, St. Louis, MO). Twenty units per milliliter each of restriction enzymes EcoRI and HindIII (Invitrogen, Paisley, United Kingdom) was added to cultures for NET digestion for 2 h at 37°C. The activity of restriction enzymes was stopped with 5 mM EDTA for 15 min at 65°C. Extracellular DNA was then quantified by using a Picogreen double-stranded DNA (dsDNA) kit (Invitrogen), in accordance with the manufacturer's instructions.

**NET-mediated bacterial killing.** Purified PMNs were placed in 24-well tissue culture plates and incubated for 30 min at 37°C in the presence and absence of the phagocytosis inhibitor cytochalasin D (10 µg/ml; Sigma). To inhibit NET-mediated bacterial killing, PMNs were incubated with DNase I (100 units/ml; Invitrogen) for 15 min prior to addition of bacteria. *B. pseudomallei* WT K96243 was then added to the PMNs at an MOI of 10, followed by continued incubation in the presence or absence of DNase I for 90 min. Infected PMNs were then lysed with 1% Triton X-100 for 10 min, and each well was scraped to recover all lysed cells. Serial dilutions of the lysates were plated to LB agar plates, and bacterial colonies were counted after 48 h of incubation.

**Phagocytosis assay by using flow cytometry.** *B. pseudomallei* WT and mutant strains were cultured overnight in LB broth and were adjusted to 1 × 10<sup>8</sup> CFU/ml in phosphate-buffered saline (PBS). Bacteria were collected by centrifugation, incubated with 1 µg/ml fluorescein isothiocyanate (FITC; Sigma) for 60 min at room temperature, and then washed three times in 1 ml of PBS. The intensity of the FITC signal due to labeled bacteria was determined prior to use. FITC-labeled bacteria at an MOI of 10 were incubated at 37°C for 90 min in 5 × 10<sup>6</sup> cells/ml of PMNs purified from healthy donors. Cells were washed twice in ice-cold PBS and then fixed in 2% (vol/vol) paraformaldehyde in PBS prior to flow cytometric analysis.

**Oxidative burst assay by using flow cytometry.** Bacteria at an MOI of 10 were added into 50 µl diluted heparinized whole blood at 37°C for 90 min, and 800 ng/ml PMA was used as a positive control. Dihydroethidium bromide (Hydroethidine; Sigma) was added to a final concentration of 2,800 ng/ml of blood at 37°C for 5 min. Cells were incubated in 2 ml fluorescence-activated cell sorter (FACS) lysing solution (BD Bioscience) to lyse red blood cells, washed twice with PBS, and fixed with 2% (vol/vol) paraformaldehyde in PBS. Following lysis and fixation, flow cytometric analysis was performed with a FACScan flow cytometer by using Cellquest software (BD Bioscience).

**Immunofluorescence assay.** PMNs from healthy subjects were incubated at a concentration of 2.5 × 10<sup>6</sup> cells/ml with medium control or *B.*



**FIG 1** Immunostaining of NETs induced by *B. pseudomallei*. (A to C) PMNs isolated from healthy control subjects were incubated with *B. pseudomallei* WT K96243 at an MOI of 10 for 90 min at 37°C and then stained for DNA with DAPI (A), histones using anti-histone H3 rabbit polyclonal antibody detected with anti-rabbit Ig Alexa Fluor 488 (B), and bacteria using anti-*B. pseudomallei* LPS antibody detected with anti-mouse Ig Alexa Fluor 568 (C). (D) Overlay of the images reveals bacteria trapped in NETs. (E to G) Representative confocal micrographs of uninfected PMNs (E) and PMNs infected with *B. pseudomallei* for 90 min at 37°C in the absence (F) and presence (G) of 100 units/ml DNase I confirm that bacteria are trapped in DNA-containing networks. For panels E to G, DNA was stained with PI and bacteria were detected with rabbit anti-*B. pseudomallei* LPS antibody detected with anti-rabbit Ig Alexa Fluor 488. Photomicrographs are of representative fields from three independent experiments. (H) The kinetics of *B. pseudomallei*-induced NET formation over time and at varied MOIs were assessed by quantifying extracellular DNA using a Picogreen dsDNA kit. Error bars indicate SEMs of three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (ANOVA and Dunnett's posttest). Magnification =  $\times 512$ ; bars = 50  $\mu\text{m}$ .

*pseudomallei* WT K96243 at an MOI of 10 (unless specified elsewhere) at 37°C for 90 min in the absence or presence of 100 units/ml of DNase I (Invitrogen). Samples were fixed with 4% (vol/vol) paraformaldehyde and blocked with 3% (wt/vol) bovine serum albumin (BSA; Sigma) in PBS for 30 min at room temperature. Bacteria were stained with a rabbit polyclonal anti-*B. pseudomallei* antibody or mouse polyclonal anti-LPS antibody, and primary antibodies were detected by mouse anti-rabbit Ig Alexa Fluor 488 or a goat anti-mouse IgG Alexa Fluor 568 antibody (Molecular Probes, Leiden, Netherlands). Histones were stained with a rabbit polyclonal anti-histone H3 antibody and a mouse anti-rabbit Ig Alexa Fluor 488 antibody (Molecular Probes) (17). Double-stranded DNA was stained with propidium iodide (PI) or 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes). Stained cells were washed and analyzed by confocal laser scanning microscopy (LSM 510 META microscope; Carl Zeiss, Germany).

**Treatment of PMNs with inhibitors.** The NADPH oxidase inhibitor diphenyleneiodonium chloride (DPI), the PI3K inhibitor wortmannin, the p38-MAPK inhibitor SB203580, and the Src family kinase inhibitor PP2 were purchased from Sigma. The chemokine CXCL8 (IL-8) was purchased from Peprotech (Rocky Hill, NJ). Briefly, PMNs were pretreated with DPI, wortmannin, SB203580, or PP2 at the doses indicated in the legend to Fig. 3 at 37°C for 90 min, prior to stimulation with 100 nM PMA, 100 nM recombinant human CXCL8 (IL-8), or *B. pseudomallei* WT K96243 at an MOI of 10 at 37°C for 90 min, and NET release was then quantified as described above.

**Statistical analysis.** Statistical analysis was performed using tests appropriate to the data set, as specified in the figure legends, and GraphPad

Prism (version 5) software (GraphPad, San Diego, CA).  $P$  values of  $\leq 0.05$  were considered statistically significant.

## RESULTS

***B. pseudomallei* induces NET formation in time- and dose-dependent manner.** We first studied whether NET formation was induced by *B. pseudomallei*. The composition of extracellular material released by *B. pseudomallei*-infected human PMNs was analyzed by confocal microscopy. We observed that *B. pseudomallei* colocalized with DNA ejected from PMNs which stained with antibody specific for histone H3 (Fig. 1A to D). The morphology and composition of the ejected material are typical of NETs. To determine whether *B. pseudomallei*-induced NETs were degraded by DNase treatment, PMNs from healthy human subjects were infected with *B. pseudomallei* in the absence or presence of DNase I enzyme. Compared to uninfected cells (Fig. 1E), propidium iodide staining of DNA revealed the release of webs of extracellular DNA containing trapped bacteria upon *B. pseudomallei* infection (Fig. 1F). Treatment of such infected PMNs with DNase I resulted in the loss of such extracellular DNA networks, and the bacteria no longer appeared to be entrapped (Fig. 1G), suggesting that released nucleotides were composed of DNA but not RNA.

The kinetics of NET formation were next studied in *B. pseudomallei*-infected PMNs over time and a range of MOIs. Extracellular DNA in culture supernatants was quantified as an



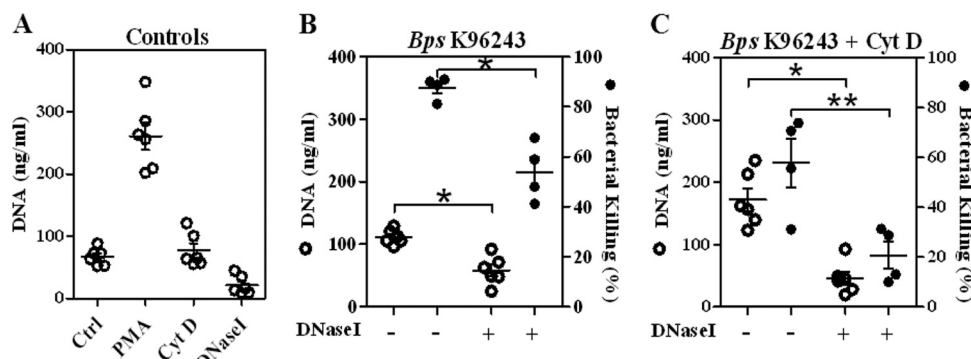


FIG 2 *B. pseudomallei* is killed by NET formation. (A) Purified PMNs pretreated with medium alone, 100 nM the mitogen PMA, 10  $\mu$ g/ml of the phagocytosis inhibitor cytochalasin D (Cyt D), or 100 units/ml of DNase I after incubation for 90 min from control assays for extracellular DNA are shown. (B and C) Release of extracellular DNA on the left y axis ( $n = 6$ ) and bacterial killing on the right y axis ( $n = 4$ ) at 90 min after infection with *B. pseudomallei* WT K96243 at an MOI of 10 are shown. (B) Cells were incubated with 100 units/ml of DNase I or medium for 15 min before the addition of bacteria. (C) Impact of cytochalasin D on extracellular DNA release and bacterial killing of *B. pseudomallei* by human PMNs in the presence or absence of DNase I is shown. The horizontal line denotes the mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (paired  $t$  test).

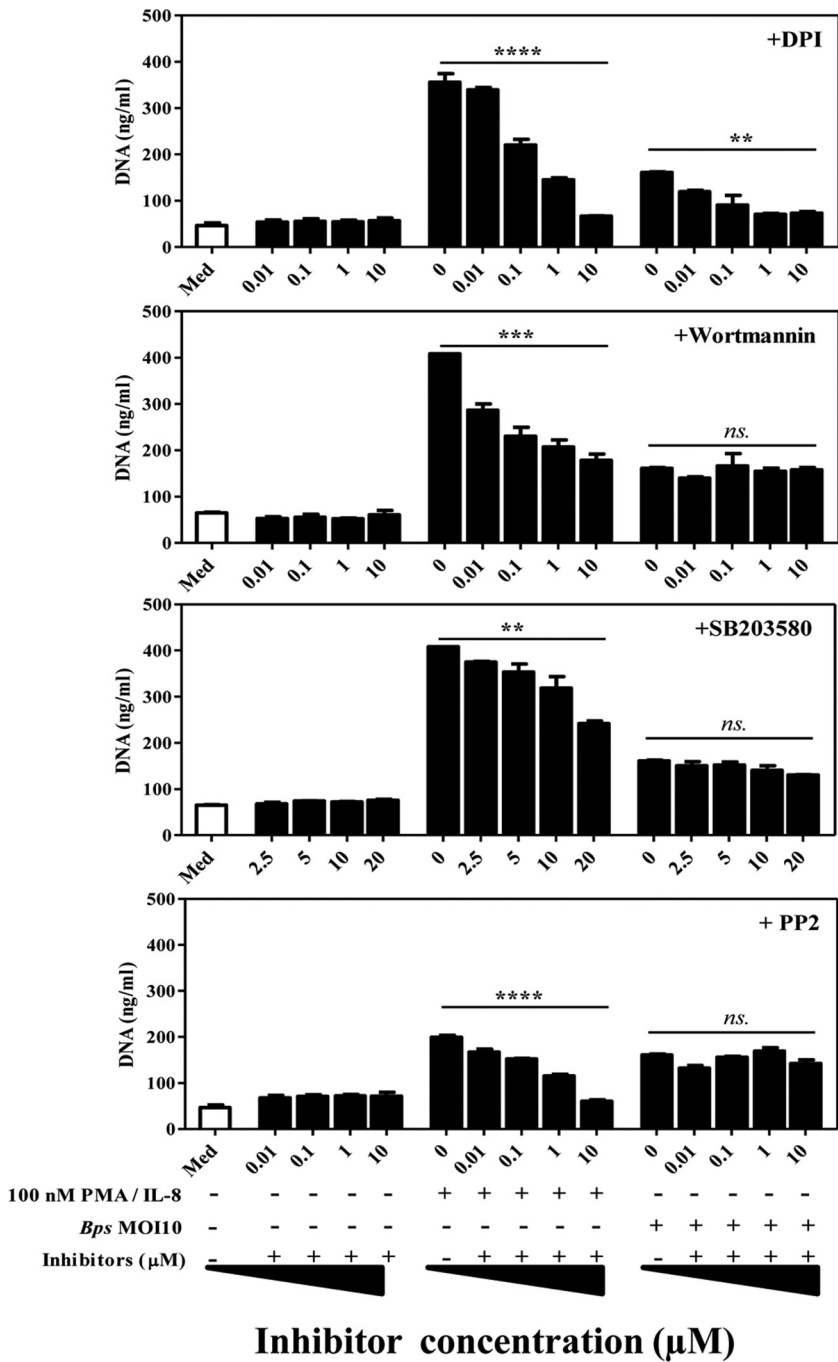
indicator of NET formation as described in Materials and Methods. Although the extent of DNA release was comparable to control levels at an MOI of 0.1 or 0.3, the amount of extracellular DNA released by infected PMNs was significantly increased at MOIs of from 1 to 10 and at 60 min postinfection and time intervals thereafter (Fig. 1H).

**NETs kill *B. pseudomallei*.** We next examined the consequences of NET release for *B. pseudomallei* by quantifying viable bacteria after infection of PMNs from healthy subjects. Under the assay conditions, stimulation of the cells with PMA elicited the release of extracellular DNA relative to the findings for PMNs in medium alone, as expected (Fig. 2A). Cytochalasin D did not affect the levels of extracellular DNA at the concentration used in subsequent studies (10  $\mu$ g/ml), and DNase I treatment was confirmed to degrade extracellular DNA (Fig. 2A). Upon infection with WT *B. pseudomallei* K96243 at an MOI of 10 (Fig. 2B), the quantity of extracellular DNA released after 90 min was significantly increased relative to that released by uninfected cells (Fig. 2A). In addition, DNase I treatment significantly degraded *B. pseudomallei*-induced extracellular DNA (Fig. 2B, left y axis). Importantly, the percentage of the inoculum killed by infected PMNs fell significantly upon DNase I treatment (Fig. 2B, right y axis), indicating that a subset of the bacteria is killed by PMNs via NET formation.

Under the assay conditions used in this study, bacteria may be killed by NETs and/or by intracellular processes. To separate these events, infected PMNs were treated with a concentration of cytochalasin D previously reported to substantially reduce entry of *Shigella flexneri* into host cells (12). Upon cytochalasin D treatment, the number of bacteria killed fell significantly from approximately 90% (1.0- $\log_{10}$ -CFU reduction) for untreated cells (Fig. 2B) to approximately 50% (0.3- $\log_{10}$ -CFU reduction) of the initial inoculum for treated cells (Fig. 2C). Cytochalasin D therefore promoted survival by preventing uptake and intracellular killing. Importantly, when the cytochalasin D-treated PMNs infected with *B. pseudomallei* were treated by DNase I, bacterial killing was significantly reduced in a manner associated with degradation of the released DNA (Fig. 2C). This further supports the conclusion that NETs have antimicrobial activity against extracellular *B. pseudomallei*.

***B. pseudomallei*-induced NET formation requires NADPH oxidase activation.** To address the mechanism of NET formation, we stimulated PMNs with the potent activator PMA and biologically relevant stimulus IL-8, as well as with *B. pseudomallei*. We then determined whether NADPH oxidase, PI3K, MAPK, and Src family kinase pathways were involved in NET formation by using the specific inhibitors DPI, wortmannin, SB203580, and PP2, respectively. Stimulation with live bacteria or PMA triggered the induction of NETs, as expected. Moreover, inhibitor pretreatment was not cytotoxic to PMNs at the concentrations used, as determined by trypan blue exclusion (data not shown), and the inhibitors did not induce the release of extracellular DNA at levels that differed from those for the control (Fig. 3). The DPI, wortmannin, and SB203580 inhibitors reduced the PMA-induced formation of NETs in a dose-dependent manner (Fig. 3), as previously reported (13, 20, 23). Moreover, PP2 inhibited IL-8-induced formation of NETs, as expected (20, 24). Though *B. pseudomallei* induced the release of extracellular DNA, this was inhibited by DPI in a dose-dependent and statistically significant manner (analysis of variance [ANOVA] and Dunnett's posttest,  $P < 0.01$ ), indicating that NADPH oxidase is required for efficient NET formation upon *B. pseudomallei* infection. Inhibitors of PI3K, MAPK, and Src family kinase signaling pathways did not significantly affect NET formation by *B. pseudomallei* (Fig. 3).

***B. pseudomallei* *bsa* and *wcbB* mutants induce elevated levels of NET formation.** Having demonstrated that wild-type *B. pseudomallei* induces NET formation by human PMNs and that such NETs exhibit bactericidal activity, we next examined the impact of mutations affecting known virulence factors of *B. pseudomallei* in these processes. Two independent *Bsa* type III secretion mutants (K96243 *bsaQ* and 10276 *bsaZ*) were used to infect PMNs in parallel with the isogenic parent strains for 90 min at an MOI of 10, and extracellular DNA was quantified. Infection by the *bsaQ* and *bsaZ* mutants caused the release of significantly more extracellular DNA relative to the amount released by the cognate parent strains (Fig. 4A). In addition, we observed that the amount of extracellular DNA released by human PMNs was significantly higher following infection by a *wcbB* mutant which lacks capsular polysaccharide I (CPS-I; a chromosome 1-encoded polymer of

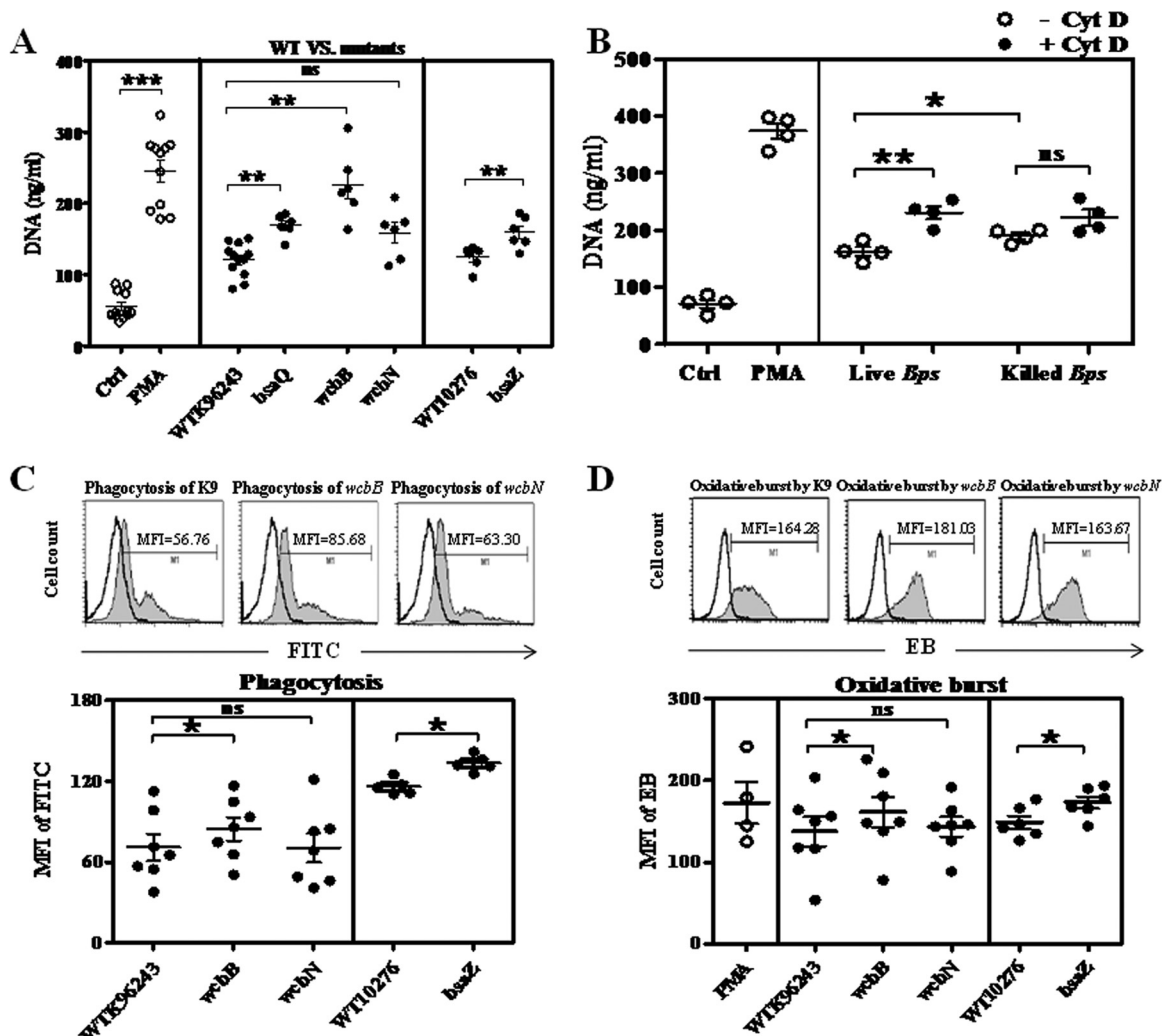


**FIG 3** NADPH oxidase is required for *B. pseudomallei*-mediated NETs. PMNs from healthy controls were pretreated for 30 min with the NADPH oxidase inhibitor DPI, the PI3K inhibitor wortmannin, the p38-MAPK inhibitor SB203580, or the Src family kinase inhibitor PP2 and were then stimulated for 90 min with PMA (100 nM for DPT, wortmannin, and SB203580 treatments), recombinant human CXCL8 (IL-8; 100 nM for PP2 treatment), or *B. pseudomallei* WT K96243 at an MOI of 10. The DNA in the supernatants was quantified as described in the text. White bars represent untreated cells (Med; medium only). The results are for data from one representative of three independent experiments with similar results. The horizontal line denotes the mean  $\pm$  SEM. \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$  (ANOVA and Dunnett's posttest).

unbranched manno-heptopyranose residues [9]) than infection by the K96243 parent strain (Fig. 4A). Upon infection by a *wcbN* mutant, which lacks the ability to synthesize GDP- $\alpha$ -D-manno-heptopyranose and retains an epitope for a capsule-specific antibody but has reduced virulence in mice (8, 9), the amount of extracellular DNA released by PMNs was

elevated relative to that released by cells infected by the parent strain, but not significantly so (Fig. 4A).

To determine if NET induction in these assays required live bacteria, PMNs were incubated with live or killed *B. pseudomallei* for 90 min in the absence or presence of 10  $\mu$ g/ml cytochalasin D. We found that killed *B. pseudomallei* significantly induced NETs



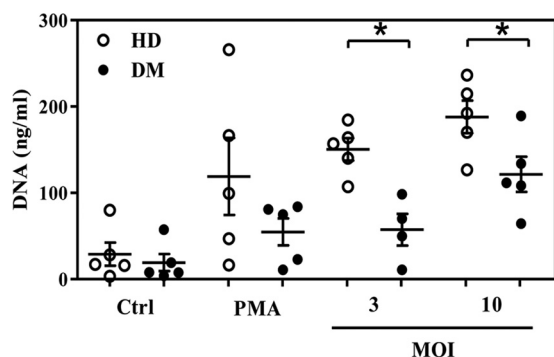
**FIG 4** Bacterial factors influence NET formation. (A) Extracellular DNA was released from PMNs from healthy subjects ( $n = 6$ ) upon infection by *B. pseudomallei* *bsa* and capsule mutants or their isogenic parent strains at an MOI of 10. (B) Healthy PMNs ( $n = 4$ ) were incubated for 90 min with live or killed WT *B. pseudomallei* K96243 at an MOI of 10 in the absence or presence of 10  $\mu$ g/ml cytochalasin D (Cyt D). (C) Isolated PMNs were incubated with medium alone or FITC-labeled live WT *B. pseudomallei* K96243 and its capsule mutant or FITC-labeled live WT *B. pseudomallei* 10276 and its *bsaZ* mutant at an MOI of 10 and analyzed by using the mean fluorescence intensity (MFI) of FITC-labeled live *B. pseudomallei*. (D) Heparinized whole-blood samples were incubated with medium alone, 800 ng/ml PMA, or live WT *B. pseudomallei* K96243 and its capsule mutants at an MOI of 10. The oxidative burst was analyzed by using the mean fluorescence intensity of ethidium bromide (EB). The horizontal line denotes the mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; ns, nonsignificant (Mann-Whitney test).

compared to live *B. pseudomallei*. However, after cytochalasin D treatment, the difference in stimulation of NET release by killed *B. pseudomallei* was not statistically significant (Fig. 4B), suggesting that NET induction could occur without the uptake of bacteria.

It has been reported that NET formation is dependent on ROS production (13). To address whether *bsa* or capsule mutations exert their effect on NET formation via effects on phagocytosis and oxidative burst, we performed flow cytometric analysis of these processes using wild-type and mutant strains. The results revealed that the *B. pseudomallei* *bsaZ* and *wcbB* mutants were phagocytosed by PMNs at a significantly higher level than the cognate parent strain (Fig. 4C). Uptake of the *wcbN* mutant was not significantly different from that of the wild type (Fig. 4C). In addition, the magnitude of the oxidative burst was found to be significantly elevated for the *bsaZ* and *wcbB* mutants compared to

that for the corresponding wild-type strains, whereas the oxidative burst induced by the *wcbN* mutant was comparable to that induced by the parent strain (Fig. 4D). Taken together with the finding that NADPH oxidase is required for full NET induction, the results indicated that *B. pseudomallei*-induced NET formation is influenced by the level of phagocytosis and oxidative burst response by human PMNs. Moreover, the data imply that the Bsa T3SS and CPS-I play a role in evading this response. Indeed, we observed that increased NET induction by the *B. pseudomallei* *bsaZ* and *wcbB* mutants was associated with increased bacterial killing of the mutants in the same experiments (see Fig. S1 in the supplemental material), and this may provide a partial explanation for the role of such factors in virulence.

**PMNs from diabetes mellitus subjects fail to form NETs.** Previously, we reported the impairment of cellular functions in



**FIG 5** PMNs from diabetes mellitus patients exhibit impaired *B. pseudomallei*-induced NET formation. PMNs isolated from healthy control subjects (HD;  $n = 5$ ) and DM patients ( $n = 5$ ), who were defined as having poor or very poor glycemic control on the basis of the levels of HbA1c (8.5 and  $>8.5\%$ , respectively), were stimulated with PMA or WT *B. pseudomallei* K96243 at an MOI of 3 or 10 for 90 min at  $37^{\circ}\text{C}$ , and then the amount of extracellular DNA was quantified by using a Picogreen kit. The horizontal line denotes the mean  $\pm$  SEM. \*,  $P < 0.05$  (Mann-Whitney test).

PMNs from human subjects with diabetes mellitus (6). PMNs from diabetic subjects were therefore compared to PMNs from healthy subjects for their ability to release extracellular DNA in response to PMA stimulation or *B. pseudomallei* infection. The results revealed that PMNs from DM patients released significantly less extracellular DNA after infection with *B. pseudomallei* at an MOI of 3 or 10 than PMNs from healthy controls (Fig. 5). In addition, PMNs from DM subjects also released significantly lower quantities of extracellular DNA upon stimulation with PMA, suggesting that the lower magnitude of the response might not be specific to their ability to respond to *B. pseudomallei*. In a further independent experiment, the reduced formation of NETs by PMNs from DM subjects compared to healthy controls was confirmed and could be associated with reduced bacterial killing under the assay conditions (see Fig. S2 in the supplemental material).

## DISCUSSION

Neutrophil extracellular traps are a recently described antimicrobial mechanism deployed by PMNs. NETs are lattices of extracellular DNA, histones, and antimicrobial proteins released upon PMN activation that ensnare and kill microorganisms (4, 13). PMNs play a key role in the control of *B. pseudomallei* infection (6), yet the ability of *B. pseudomallei* to induce or evade NET-mediated killing is ill defined. Here, we demonstrated that *B. pseudomallei* infection of human PMNs elicits the release of DNA- and histone-rich NETs from naïve PMNs which are not exposed to any other stimuli. Moreover, the amount of DNA released increased in a time- and dose-dependent manner. DNase I treatment of infected PMNs was observed to degrade the extracellular DNA and enhance bacterial survival, indicating that NETs have bactericidal activity against *B. pseudomallei*. Cytochalasin D treatment indicated that a subset of *B. pseudomallei* is killed extracellularly by PMNs, and such killing was again reduced by dissociation of NETs with DNase I.

NADPH oxidase is a membrane-bound enzyme complex found in the plasma membrane as well as in phagosomal membranes and reported to trigger NETosis, an unique cell death pathway which is distinct from both apoptosis and necrosis (2, 13). It is

a highly regulated complex that is capable of producing large amounts of superoxide that lead to an oxidative burst (2). The signaling pathways contributing to *B. pseudomallei*-induced NET formation were investigated by using the inhibitors DPI, wortmannin, SB203580, and PP2. Our data indicate that NADPH oxidase activity is required for NET formation but suggest that PI3K, MAPK, and Src family kinases are not essential for this process. DPI has been established to abolish NADPH oxidase-mediated ROS formation at the concentration used (11); however, we cannot be certain that this directly impairs NET formation, as superoxide generated by NADPH oxidase (Nox) family members may activate other flavoenzymes, such as xanthine oxidase and NO synthase, to generate further superoxide (40).

Gram-negative bacterial pathogens of several genera use type III protein secretion to alter the function of phagocytes and antigen-presenting cells. Such interference arises from the injection of bacterial proteins directly into target cells, which then subvert or inhibit cellular processes to the benefit of the pathogen. Two independent mutants lacking the function of the Bsa T3SS elicited the release of higher levels of extracellular DNA than the parent strain. This was associated with increased uptake of the *bsaZ* mutant into PMNs and increased production of ROS. Though we speculate that the differences in NET release by Bsa mutants may be due to differences in the level of phagocytosis, it is possible that bacterial inhibition of NADPH oxidase assembly or activity may contribute to the effect. Indeed, it is known that *Salmonella* uses a T3SS encoded by *Salmonella* pathogenicity island 2 (SPI-2) to inhibit NADPH oxidase assembly on the membrane of *Salmonella*-containing vacuoles, leading to reduced production of ROS and improved intracellular net replication (14, 36). Moreover, the *Yersinia pestis* T3SS inhibits the respiratory burst of human PMNs and contributes to extracellular survival via the injection of Yop proteins, albeit intracellular survival of *Y. pestis* appears to be independent of T3SS-mediated inhibition of neutrophil ROS production (26, 27). In contrast to these organisms, *B. pseudomallei* has been reported to rapidly escape from phagosomes, and further studies are needed to determine if the events described above apply during interactions with primary human PMNs. Moreover, it may be difficult to separate the role of the Bsa needle in contact-mediated lysis of the phagosomal membrane and escape to the cytosol from the effects of Bsa-secreted effectors on cellular activities.

It has previously been reported that NETs degrade IpaB of the *Shigella* T3SS by proteolysis, thereby disarming the secretion apparatus (12). *In vitro* *B. pseudomallei* secretes a number of extracellular enzymes that have the potential to counteract activities presented in NETs (1). It is conceivable that such products may counteract the bactericidal activity of NETs. For example, expression of a surface endonuclease enables *S. pneumoniae* to degrade the DNA scaffold of NETs and escape killing (3). While a DNase has been predicted in *B. pseudomallei* (19), evidence that this activity is secreted or surface associated is lacking.

We also established that capsule-deficient mutants of *B. pseudomallei* induce elevated levels of extracellular DNA upon infection of PMNs. The response was greater with a *wcbB* mutant completely lacking the polymer of unbranched manno-heptopyranose residues than with a *wcbN* mutant that retains a capsule-specific epitope but whose virulence is attenuated (9). In common with other encapsulated bacterial pathogens, it appears that the *B. pseu-*



*domallei* capsule interferes with phagocytosis by human PMNs, as a *wcbB* mutant was taken up in higher numbers than either the parent strain or the *wcbN* mutant, which retains some capsule. Following uptake, the *wcbB* mutant induced a stronger oxidative burst, which, taken together with the role of NADPH oxidase in NET formation, may explain why the mutant elicited the release of elevated levels of extracellular DNA.

In a previous report, we demonstrated that PMNs from diabetes mellitus patients are impaired in their ability to phagocytose *B. pseudomallei* and to produce an oxidative burst to kill intracellular bacteria (6). In addition to finding that NET induction can be influenced by bacterial factors, it is evident from the present study that PMNs from diabetes mellitus patients are impaired in their ability to release extracellular DNA in response to *B. pseudomallei* infection. Reduced NET formation by PMNs from DM subjects was associated with reduced bacterial killing relative to that by PMNs from healthy controls; however, we cannot preclude the possibility that this may be due to impaired uptake or ROS production. Indeed, there was no significant difference in DNA release between PMA-stimulated PMNs from healthy and diabetic subjects, suggesting that PMNs from DM patients are not defective in NET formation *per se*. Taken together with other defects in PMN functions, the impairment of NET release may provide a partial explanation for the elevated susceptibility of diabetes mellitus patients to clinical melioidosis and other forms of sepsis.

## ACKNOWLEDGMENTS

D. Riyapa is supported by the Joint Ph.D. Program under the Strategic Scholarships for Frontier Research Network, Office of the Higher Education Commission, Thailand. The work was also supported in part by the National Institutes of Health, National Institute of Allergy and Infectious Diseases (grant number U01 AI082110).

We thank Chidchamai Kewcharoenwong for her assistance with flow cytometry.

We have no commercial or other associations that might pose a conflict of interest.

## REFERENCES

- Ashdown LR, Koehler JM. 1990. Production of hemolysin and other extracellular enzymes by clinical isolates of *Pseudomonas pseudomallei*. J. Clin. Microbiol. 28:2331–2334.
- Babior BM. 1999. NADPH oxidase: an update. Blood 93:1464–1476.
- Beiter K, et al. 2006. An endonuclease allows *Streptococcus pneumoniae* to escape from neutrophil extracellular traps. Curr. Biol. 16:401–407.
- Brinkmann V, et al. 2004. Neutrophil extracellular traps kill bacteria. Science 303:1532–1535.
- Brinkmann V, Zychlinsky A. 2007. Beneficial suicide: why neutrophils die to make NETs. Nat. Rev. Microbiol. 5:577–582.
- Chanchamroen S, Kewcharoenwong C, Sussaengrat W, Ato M, Lertmemongkolchai G. 2009. Human polymorphonuclear neutrophil responses to *Burkholderia pseudomallei* in healthy and diabetic subjects. Infect. Immun. 77:456–463.
- Cheng AC, Currie BJ. 2005. Melioidosis: epidemiology, pathophysiology, and management. Clin. Microbiol. Rev. 18:383–416.
- Cuccui J, et al. 2007. Development of signature-tagged mutagenesis in *Burkholderia pseudomallei* to identify genes important in survival and pathogenesis. Infect. Immun. 75:1186–1195.
- Cuccui J, et al. 2012. Characterization of the *Burkholderia pseudomallei* K96243 capsular polysaccharide I coding region. Infect. Immun. 80:1209–1221.
- Easton A, Haque A, Chu K, Lukaszewski R, Bancroft GJ. 2007. A critical role for neutrophils in resistance to experimental infection with *Burkholderia pseudomallei*. J. Infect. Dis. 195:99–107.
- Ellis JA, Mayer SJ, Jones OT. 1988. The effect of the NADPH oxidase inhibitor diphenyleneiodonium on aerobic and anaerobic microbicidal activities of human neutrophils. Biochem. J. 251:887–891.
- Francois M, Le Cabec V, Dupont MA, Sansonetti PJ, Maridonneau-Parini I. 2000. Induction of necrosis in human neutrophils by *Shigella flexneri* requires type III secretion, IpaB and IpaC invasins, and actin polymerization. Infect. Immun. 68:1289–1296.
- Fuchs TA, et al. 2007. Novel cell death program leads to neutrophil extracellular traps. J. Cell Biol. 176:231–241.
- Gallois A, Klein JR, Allen LA, Jones BD, Nauseef WM. 2001. Salmonella pathogenicity island 2-encoded type III secretion system mediates exclusion of NADPH oxidase assembly from the phagosomal membrane. J. Immunol. 166:5741–5748.
- Holden MT, et al. 2004. Genomic plasticity of the causative agent of melioidosis, *Burkholderia pseudomallei*. Proc. Natl. Acad. Sci. U. S. A. 101:14240–14245.
- Leelarasamee A. 2004. Recent development in melioidosis. Curr. Opin. Infect. Dis. 17:131–136.
- Li P, et al. 2010. PAD4 is essential for antibacterial innate immunity mediated by neutrophil extracellular traps. J. Exp. Med. 207:1853–1862.
- Limmathurtsakul D, et al. 2006. Risk factors for recurrent melioidosis in northeast Thailand. Clin. Infect. Dis. 43:979–986.
- MacLea KS, Krieser RJ, Eastman A. 2003. A family history of deoxyribonuclease II: surprises from *Trichinella spiralis* and *Burkholderia pseudomallei*. Gene 305:1–12.
- Marcos V, et al. 2010. CXCR2 mediates NADPH oxidase-independent neutrophil extracellular trap formation in cystic fibrosis airway inflammation. Nat. Med. 16:1018–1023.
- Muangsoont V, et al. 2008. Inactivation of *Burkholderia pseudomallei* bsaQ results in decreased invasion efficiency and delayed escape of bacteria from endocytic vesicles. Arch. Microbiol. 190:623–631.
- Nathan C. 2006. Neutrophils and immunity: challenges and opportunities. Nat. Rev. Immunol. 6:173–182.
- Remijsen Q, et al. 2011. Neutrophil extracellular trap cell death requires both autophagy and superoxide generation. Cell Res. 21:290–304.
- Sai J, Raman D, Liu Y, Wikswo J, Richmond A. 2008. Parallel phosphatidylinositol 3-kinase (PI3K)-dependent and Src-dependent pathways lead to CXCL8-mediated Rac2 activation and chemotaxis. J. Biol. Chem. 283:26538–26547.
- Simpson AJ, Newton PN, Chierakul W, Chaowagul W, White NJ. 2003. Diabetes mellitus, insulin, and melioidosis in Thailand. Clin. Infect. Dis. 36:e71–e72.
- Spinner JL, Cundiff JA, Kobayashi SD. 2008. *Yersinia pestis* type III secretion system-dependent inhibition of human polymorphonuclear leukocyte function. Infect. Immun. 76:3754–3760.
- Spinner JL, et al. 2010. Neutrophils are resistant to *Yersinia* YopJ/P-induced apoptosis and are protected from ROS-mediated cell death by the type III secretion system. PLoS One 5:e9279. doi:10.1371/journal.pone.0009279.
- Stevens MP, et al. 2004. Attenuated virulence and protective efficacy of a *Burkholderia pseudomallei* bsa type III secretion mutant in murine models of melioidosis. Microbiology 150:2669–2676.
- Stevens MP, et al. 2002. An Inv/Mxi-Spa-like type III protein secretion system in *Burkholderia pseudomallei* modulates intracellular behaviour of the pathogen. Mol. Microbiol. 46:649–659.
- Suparak S, et al. 2005. Multinucleated giant cell formation and apoptosis in infected host cells is mediated by *Burkholderia pseudomallei* type III secretion protein BipB. J. Bacteriol. 187:6556–6560.
- Suputtamongkol Y, et al. 1999. Risk factors for melioidosis and bacteremic melioidosis. Clin. Infect. Dis. 29:408–413.
- Takami M, Terry V, Petruzzelli L. 2002. Signaling pathways involved in IL-8-dependent activation of adhesion through Mac-1. J. Immunol. 168:4559–4566.
- Ulrich RL, DeShazer D. 2004. Type III secretion: a virulence factor delivery system essential for the pathogenicity of *Burkholderia mallei*. Infect. Immun. 72:1150–1154.
- Urban CF, Lourido S, Zychlinsky A. 2006. How do microbes evade neutrophil killing? Cell. Microbiol. 8:1687–1696.
- Urban CF, Reichard U, Brinkmann V, Zychlinsky A. 2006. Neutrophil extracellular traps capture and kill *Candida albicans* yeast and hyphal forms. Cell. Microbiol. 8:668–676.
- Vazquez-Torres A, et al. 2000. Salmonella pathogenicity island 2-dependent evasion of the phagocyte NADPH oxidase. Science 287:1655–1658.
- Warawa JM, Long D, Rosenke R, Gardner D, Gherardini FC. 2009. Role

- for the *Burkholderia pseudomallei* capsular polysaccharide encoded by the wcb operon in acute disseminated melioidosis. *Infect. Immun.* 77:5252–5261.
38. Wartha F, et al. 2007. Capsule and D-alanylated lipoteichoic acids protect *Streptococcus pneumoniae* against neutrophil extracellular traps. *Cell. Microbiol.* 9:1162–1171.
  39. White NJ. 2003. Melioidosis. *Lancet* 361:1715–1722.
  40. Wind S, et al. 2010. Comparative pharmacology of chemically distinct NADPH oxidase inhibitors. *Br. J. Pharmacol.* 161:885–898.
  41. Yost CC, et al. 2009. Impaired neutrophil extracellular trap (NET) formation: a novel innate immune deficiency of human neonates. *Blood* 113:6419–6427.